Reference: *Biol. Bull.* 177: 247–253. (October, 1989)

Cryopreservation of Spermatophores and Seminal Plasma of the Edible Crab *Scylla serrata*

C. JEYALECTUMIE AND T. SUBRAMONIAM

Unit of Invertebrate Reproduction, Department of Zoology, University of Madras, Guindy Campus, Madras 600 025, India

Abstract. This paper describes the development of a suitable biotechnology to cryopreserve the spermatophores of the edible crab *Scylla serrata* in a viable condition. Three temperatures (−4°C, −79°C, and −196°C) were chosen to preserve the spermatophores and seminal plasma, collected from the middle region of vas deferens of mature male crabs, for 30 days. Sperm viability was determined by the eosin-nigrosin dye exclusion method, as applied to entire spermatophores. Of the three temperatures tested, the maximum percentage of sperm viability was obtained at −196°C, whereas it significantly decreased at −4°C. Biochemical alterations of the major substrates such as proteins, carbohydrates, and lipids, as well as the enzyme Lactate Dehydrogenase (LDH) occurred only at −4°C, reflecting their use in the metabolic activities of the spermatozoa contained within the spermatophores. At −4°C, the TCA-soluble total free sugars increased in correspondence with a decline in the bound sugars, suggesting that the latter may be used rapidly during sperm storage. Our data also suggest that, at −79°C and −196°C, the frozen spermatozoa retain viability but do not exhibit metabolic activity.

To investigate the role of cryoprotectants in preventing damage to the sperm cells/spermatophores during cryopreservation, four cryoprotectants, glycerol, dimethyl sulfoxide (DMSO), trehalose, and DMSO + trehalose combination, were tested. Using the phosphate buffer as the standard diluent, glycerol gave the best result. When used alone, trehalose gave only a low sperm survival, but in combination with DMSO, it gave an increased viability that equalled the result with glycerol. We recommend that glycerol is the best cryoprotectant inasmuch as the biochemical alterations in the glycerol medium is less, compared to that of DMSO + trehalose. When used alone, DMSO may be more toxic to the sperm cells as it gave a low viability value even at −196°C and −79°C.

Received 8 December 1988; accepted 31 July 1989.
DMSO—Dimethyl sulfoxide.

Introduction

Cryopreservation of gametes is a common method practiced in conjunction with artificial insemination in mammals (Leverage *et al.*, 1972). Many attempts have also been made in this respect to cryopreserve spermatozoa of the teleost fishes (Horton and Ott, 1976). Although decapod crustaceans have become important in aquaculture in recent years, cryopreservation techniques have not been employed for *in vitro* fertilization in prawns, lobsters, or crabs. However, using electroejaculation techniques, extraction of intact spermatophores and their attachment onto females have been accomplished in several decapods like shrimps and lobsters (Chow *et al.*, 1985; Ishida *et al.*, 1986). On the other hand, no work has been done on the artificial insemination or *in vitro* fertilization of crabs. Brachyuran crabs produce simple spermatophores that are carried in the fluid medium of seminal plasma. Therefore, any attempt to cryopreserve male gametes will include both the spermatophores and the seminal plasma. Previous studies on the seminal plasma of crustaceans such as crabs (Jeyalectumie and Subramoniam, 1987) and cirripede (Barnes, 1962) indicated a similarity, in terms of providing energy-yielding substrates, with the semen of mammals. The objective of the present study was to determine a suitable temperature and an extender to cryopreserve the spermatophores as well as seminal plasma of the crab, *Scylla serrata*. Fluctuations in the biochemical composition of the spermatophores and seminal plasma during cryopreservation at selected subzero temperature conditions

were also followed. LDH enzyme showed significant fluctuation during spermatophore storage in the crab, *Paratelphusa hydrodromous* (Jeyalectumie and Subramoniam, 1987) and hence, in the present study, fluctuations in the LDH activity of seminal plasma of *Scylla serrata* during cryopreservation were also determined.

Materials and Methods

Collection of sample

Seminal plasma containing vesiculate spermatophores was collected in a petri dish after puncturing the thin wall of the mid vas deferens. The samples collected were used for the cryopreservation studies.

Initial cooling, dilution, and freezing

The diluent for spermatophore preservation was prepared, according to Behlmer and Brown (1984), immediately before use by mixing 25 ml of 0.4 *M* NaCl/0.1 *M* glycine, 4 ml of 0.028 M NaH_2PO_4/0.072 *M* Na_2HPO_4, and 5 ml of glycerol. Apart from this, three other diluents were prepared by substituting glycerol with trehalose (0.25 *M*), DMSO (5%), and a combination of DMSO (5%) and trehalose (0.25 *M*). These are the cryoprotectants commonly used for various biological systems (Lovelock and Polge, 1954; Hughes, 1973; Asahina and Takahashi, 1978; Zell *et al.*, 1979; Behlmer and Brown, 1984; Chow *et al.*, 1985; Stephens, 1986; Anchordogy *et al.*, 1988). Analytical grade glycerol, trehalose, and DMSO were purchased from Sarabai Chemicals (India), BDH (England), and SD's (India), respectively.

The freshly collected sample was diluted to 20% spermatophore suspension by mixing four volumes of diluent to one volume of seminal plasma. Similarly, the spermatophoric suspensions in four different diluents were prepared separately. After dilution, the seminal plasma was aspirated into 0.5-ml semen storage straws having one end sealed. After filling, the open side of the straw was sealed with polyvinyl alcohol. Immediately after this process, all the straws containing the sample were kept for equilibration at 4°C for 16 h. Prior to freezing at −196°C in liquid nitrogen, the straws were kept exposed to the liquid nitrogen vapor for an hour and then immersed completely in the liquid nitrogen. Similarly, for −79°C, the straws were exposed to gaseous Co_2 for an hour, and then placed directly onto the dry ice. For −4°C, the straws were kept inside the freezer. All the above samples were stored for 30 days. Fresh, unfrozen semen, with appropriate diluents, were used as a control.

Our attempts to freeze the seminal plasma without adding the diluent in the three temperature conditions did not yield fruitful results. The 'dry' seminal plasma becomes coagulated after its transfer to the subzero condition. A similar problem was also mentioned by Asahina and Takahashi (1978) and Yoo *et al.* (1987), who found that removal of cryoprotectant led to agglutination of the spermatozoa of both sea urchins and salmon. Storing semen in diluent without adding any cryoprotectant resulted in the clumping of the spermatophores after thawing, thus precluding the preparation of good smear for the viability study. Moreover, in the previous studies on cryopreservation of male gametes in fishes and invertebrates such as horseshoe crabs, molluscs, and sea urchins, only the pre-frozen semen has been used as the control (Asahina and Takahashi, 1978; Zell *et al.*, 1979; Withler, 1982; Behlmer and Brown, 1984; Kurokura *et al.*, 1986).

Thawing

Thawing was accomplished by immersing the straws containing frozen seminal plasma in tap water (room temperature). The thawed seminal plasma was collected from the straws into clean test tubes by cutting open both the sealed ends.

Evaluation of viability of cryopreserved spermatophores

To evaluate sperm viability inside the spermatophore, the eosin-nigrosin staining method of Zaneveld and Polakoski (1977) was used. A smear was prepared by mixing one drop of thawed seminal plasma with one drop of 0.5% eosin and two drops of 10% nigrosin. These slides, after being air-dried, were examined using a brightfield microscope. Care was taken to complete the observation within 2 min of smear preparation. Dead sperm cells inside the spermatophores appeared pink, whereas live cells were unstained against a red background of nigrosin. For each observation, about 300–400 spermatophores were counted in a given square area. The validity of eosin-nigrosin staining method was tested using the sperm cells, intentionally killed by exposing them to room temperature for 30 min.

Biochemical analysis

Protein was estimated by the method of Lowry *et al.* (1951). The protein in 0.2 ml of diluted seminal plasma was precipitated using 10% TCA. Using bovine serum albumin as a standard, the absorbancy was recorded at 500 nm. Total free sugars (TFS) were estimated by the method of Roe (1955). Protein-bound sugars (PBS) and glycogen were estimated following the method of Caroll *et al.* (1956). After hydrolyzing the sample to release the bound sugars in 1N H_2SO_4 at 95°C, the same was treated with anthrone reagent. Glycogen was precipitated by ethanol from the supernatant obtained during protein precipitation and was dissolved in 0.5 ml distilled water and

Table I

Cryopreservation experiment on seminal plasma of Scylla serrata *with different cryoprotectants: evaluation of spermatophore viability (percentage) (mean ± S.E.)*

		Glycerol			DMSO			Trehalose			DMSO-trehalose		
Temperature		P.F	5th day	30th day	P.F	5th day	30th day	P.F	5th day	30th day	P.F	5th day	30th day
−196°C	$\bar{X}$	98.27	97.46	95.29[n]	96.96	94.80	88.99[a]	98.23	95.69	88.67[a]	98.39	97.08	94.25[n]
	S.E.	±1.217	±1.377	±1.366	±0.494	±0.425	±0.852	±0.503	±0.563	±0.676	±0.892	±0.881	±0.012
−79°C	$\bar{X}$	96.38	95.57	93.18[n]	96.98	93.40	88.76[a]	97.75	94.98	87.16[a]	97.56	96.14	92.98[n]
	S.E.	±0.858	±0.646	±1.005	±0.645	±1.022	±0.942	±0.694	±0.592	±0.489	±1.155	±1.200	±1.146
−4°C	$\bar{X}$	98.27	93.27	80.37[a]	96.96	88.19	66.75[a]	98.23	86.17	66.44[a]	98.39	92.00	78.62[a]
	S.E.	±1.217	±0.636	±1.081	±0.494	±0.965	±1.176	±0.503	±1.301	±1.602	±0.892	±0.811	±0.905

P.F. = Prefreeze. No. of observation (n) = 6. a: $P < 0.001$. n: not significant.

then treated with anthrone reagent. The color developed for PBS and glycogen was recorded as for TFS.

Total lipids were estimated using sulphophospho-vanillin method (Barnes and Blackstock, 1973), after extracting the lipid in chloroform:methanol mixture by the method of Folch *et al.* (1957). The above results were expressed in mg/ml seminal plasma.

LDH enzyme activity was measured according to Yoshida and Freese (1975), which is based on the lactate formed from pyruvate, by the oxidation of NADH. The units of enzyme per ml reaction mixture contained in a silica cell with 1 cm light path were calculated from the rate of absorbancy change at 340 nm, by using the millimolar extinction coefficient of 6.22, and expressed in units/mg protein. One unit of enzymatic activity represents the conversion of 1 μmole of NADH per minute. The enzyme protein was also assayed following Lowry *et al.* (1951).

All results were tested for significance using analysis of variance (ANOVA) and least significant difference (LSD) (Snedecor and Cochran, 1967; Winer, 1971; Zar, 1974). Arcsin transformation for proportions were used.

Results

Viability of cryopreserved spermatophores

Results of sperm (spermatophore) viability at −196°C, −79°C, and −4°C are presented in Table I. The viability test was made with every 5-day interval, but the results are given only for the 5th and 30th days for brevity. The change in sperm viability was also not markedly different between the 10th and 30th day. The viability of sperm in 30 days of storage was fairly high, but it showed a range from 95% (at −196°C) to 80% (at −4°C). This high percentage of viability was obtained in samples containing glycerol as the cryoprotectant. However, in DMSO, the viability was reduced to 89–67%. A similar trend was observed when trehalose was used as the cryoprotectant. Conversely, when DMSO + trehalose combination was used, a higher percentage of viability (between 94% and 79%) was obtained. Of the three temperatures tested, sperm viability was poor at −4°C, whereas at −196°C, sperm viability was maximum.

Biochemical analysis

The results are shown in Table II. Statistically, there was a significant difference between the three temperatures ($P < 0.001$); the LSD revealed that at −196°C, the decrease of the biochemical components was less than at −79°C and −4°C. For protein, total free sugars, glycogen, and LDH, there was no significant difference between −196°C and −79°C, whereas lipid and protein-bound sugars showed a significant difference between these two temperatures. However, at −4°C, the decrease of PBS is highly significant ($P < 0.001$); whereas glycogen and lipid do not show any significant decrease except in DMSO for glycogen ($P < 0.005$) and in trehalose for lipid ($P < 0.001$) at this temperature.

Protein, glycogen, lipid, and LDH did not show any significant difference between glycerol and DMSO + trehalose media; whereas PBS significantly decreased in DMSO + trehalose medium when compared to glycerol. Interestingly, the TFS showed a significant increase in glycerol and DMSO media at −4°C. At this temperature, TFS increased from a control (pre-freeze) value of 6.563 mg to 8.346 mg/ml in glycerol, and from 4.268 mg to 5.563 mg/ml in DMSO on the 30th day. That this increase may be due to a release of free sugars from the bound sugars was shown from a corresponding decrease of their value from 10.257 mg of the control to 6.707 mg/ml in glycerol and 7.646 mg to 3.215 mg/ml in DMSO on the 30th day (Table II). However, in media containing trehalose, there was very little difference in value of TFS between the control and the preserved seminal plasma.

Table II

Fluctuation in biochemical components of cryopreserved seminal plasma of Scylla serrata *at three different temperatures and with different cryoprotectants (mean ± S.E.)*

		Glycerol			DMSO			Trehalose			DMSO-trehalose		
Components		P.F	5th day	30th day	P.F	5th day	30th day	P.F	5th day	30th day	P.F	5th day	30th day
Protein (mg/ml)													
−196°C	$\bar{X}$	312.348	306.375	300.913^{n}	214.863	204.152	180.390^{n}	292.985	276.670	248.985^{n}	298.713	292.437	281.920^{n}
	S.E.	±12.877	±11.761	±13.934	±11.754	±11.745	±13.383	±11.496	±17.370	±8.982	±14.137	±13.318	±12.468
−79°C	$\bar{X}$	227.928	221.695	218.715^{n}	294.230	265.078	244.890^{n}	372.875	343.360	315.848^{n}	395.783	386.075	379.650^{n}
	S.E.	±12.295	±12.649	±12.771	±11.167	±12.647	±11.694	±10.033	±12.696	±10.396	±10.534	±11.209	±9.185
−4°C	$\bar{X}$	312.348	277.997	224.350^{d}	214.863	191.912	115.188^{a}	292.985	261.523	160.512^{a}	298.713	274.333	194.753^{a}
	S.E.	±12.877	±20.041	±10.961	±11.754	±7.162	±5.919	±11.496	±14.748	±8.243	±14.137	±11.046	±8.346
LDH (Units/mg protein)													
−196°C	$\bar{X}$	91.118	86.047	85.990^{n}	68.372	64.095	60.215^{n}	95.193	90.752	80.705^{n}	99.552	96.568	93.228^{n}
	S.E.	±2.176	±2.517	±3.068	±2.339	±2.853	±3.611	±3.989	±4.202	±3.638	±4.731	±4.231	±4.543
−79°C	$\bar{X}$	89.547	86.715	84.687^{n}	78.670	73.755	67.662^{n}	94.992	89.483	78.870^{n}	91.553	88.512	85.957^{n}
	S.E.	±1.789	±1.802	±1.915	±3.788	±3.753	±3.302	±3.549	±3.290	±3.597	±2.000	±2.163	±1.697
−4°C	$\bar{X}$	91.118	79.555	57.227^{a}	68.372	52.543	32.980^{a}	95.193	76.357	41.267^{a}	99.552	84.340	60.583^{a}
	S.E.	±2.176	±4.507	±5.677	±2.339	±3.789	±3.677	±3.989	±2.689	±1.674	±4.731	±2.967	±5.056
Total free sugars (mg/ml)													
−196°C	$\bar{X}$	6.563	6.337	5.966^{n}	4.268	4.069	3.588^{n}	446.672	439.036	423.371^{n}	409.324	404.885	397.177^{n}
	S.E.	±0.284	0.276	±0.289	±0.238	±0.241	±0.243	±14.069	±14.284	±12.259	±6.869	±7.899	±6.954
−79°C	$\bar{X}$	5.110	4.919	4.628^{n}	3.801	3.633	3.229^{n}	442.540	430.845	411.262^{n}	403.074	387.748	385.254^{n}
	S.E.	±0.226	±0.211	±0.222	±0.156	±0.181	±0.169	±13.451	±15.676	±13.393	±11.485	±12.478	±12.769
−4°C	$\bar{X}$	6.563	7.096	8.346^{a}	4.268	4.542	5.563^{a}	446.672	448.649	450.935^{n}	409.324	410.327	412.085^{n}
	S.E.	±0.284	±0.224	±0.115	±0.238	±0.189	±0.154	±14.069	±16.434	±20.782	±6.869	±6.410	±7.397
Protein bound sugars (mg/ml)													
−196°C	$\bar{X}$	10.257	9.992	9.569^{n}	7.646	7.350	6.726^{n}	15.294	14.688	13.828^{n}	15.012	14.433	13.953^{n}
	S.E.	±0.328	±0.323	±0.331	±0.434	±0.382	±0.443	±0.507	±0.440	±0.425	±0.609	±0.583	±0.587
−79°C	$\bar{X}$	10.193	9.868	9.434^{n}	6.913	6.666	6.043^{n}	14.669	13.801	12.896^{n}	12.075	11.606	10.654^{n}
	S.E.	±0.424	±0.421	±0.394	±0.301	±0.312	±0.338	±0.366	±0.492	±0.429	±0.316	±0.307	±0.454
−4°C	$\bar{X}$	10.257	9.062	6.707^{a}	7.646	6.303	3.215^{a}	15.294	12.141	7.440^{a}	15.012	12.588	8.013^{a}
	S.E.	±0.328	±0.304	±0.223	±0.434	±0.414	±0.423	±0.507	±0.485	±0.474	±0.609	±0.562	±0.490
Glycogen (mg/ml)													
−196°C	$\bar{X}$	0.478	0.465	0.436^{n}	0.579	0.533	0.496^{n}	1.310	1.248	1.135^{n}	2.177	2.120	1.933^{n}
	S.E.	±0.021	±0.024	±0.020	±0.019	±0.018	±0.020	±0.222	±0.233	±0.238	±0.219	±0.223	±0.235
−79°C	$\bar{X}$	0.458	0.435	0.411^{n}	0.649	0.612	0.548^{n}	2.265	2.148	1.894^{n}	2.175	2.048	1.853^{n}
	S.E.	±0.021	±0.017	±0.012	±0.029	±0.032	±0.028	±0.200	±0.225	±0.232	±0.164	±0.162	±0.160
−4°C	$\bar{X}$	0.478	0.452	0.381^{n}	0.579	0.515	0.415^{b}	1.310	1.205	0.945^{n}	2.177	1.945	1.611^{n}
	S.E.	±0.021	±0.027	±0.021	±0.019	±0.023	±0.022	±0.222	±0.232	±0.189	±0.219	±0.220	±0.242
Lipid (mg/ml)													
−196°C	$\bar{X}$	11.270	11.062	10.642^{n}	8.161	7.859	7.082^{n}	9.811	9.425	8.570^{n}	10.070	9.883	9.303^{n}
	S.E.	±0.472	±0.477	±0.461	±0.514	±0.468	±0.459	±0.407	±0.362	±0.344	±0.509	±0.478	±0.524
−79°C	$\bar{X}$	11.152	10.836	10.248^{n}	8.494	7.849	6.985^{n}	8.388	7.801	6.886^{n}	11.925	11.614	10.833^{n}
	S.E.	±0.538	±0.529	±0.513	±0.484	±0.558	±0.517	±0.590	±0.568	±0.613	±0.458	±0.434	±0.414
−4°C	$\bar{X}$	11.270	10.676	9.502^{n}	8.161	7.385	5.946^{n}	9.811	9.046	6.742^{a}	10.070	9.419	8.040^{n}
	S.E.	±0.472	±0.453	±0.467	±0.514	±0.611	±0.526	±0.407	±0.407	±0.481	±0.509	±0.532	±0.513

P.F. = Prefreeze; No. of observation (n) = 6; a: $P < 0.001$; b: $P < 0.005$; d: $P < 0.05$; n: not significant.

Table III

Conditions of cryopreservation of male gametes of Arthropoda

Sl. no.	Species	Cryoprotectant	Temperature	Preservation period	Percentage of survival	Method of testing viability	References
	Decapod Crustacea						
1.	*Sicyonia ingentis*	DMSO + Trehalose	−196°C	2 months	60–70%	Acrosome reaction	Anchordogy *et al.*, 1987
2.	*S. ingentis*	Trehalose, Sucrose, Proline, Glycerol, DMSO	−196°C	1 month	56%	Acrosome reaction	Anchordogy *et al.*, 1988
3.	*Macrobrachium rosenbergii*	Glycerol	−196°C	31 days	53%	Fertility	Chow *et al.*, 1985
4.	*Scylla serrata*	Glycerol, DMSO, Trehalose, DMSO + Trehalose	−196°C −79°C −4°C	30 days	95% (Glycerol, DMSO + Trehalose) 89% (DMSO, Trehalose)	Eosin dye exclusion	Present study
	Insecta						
5.	*Apis mellifera*	Glycerol, Seminal vesicle fluid, Spermathecal fluid	−79°C	16 days	50%	Motility	Sawada and Chang, 1964
	Arachnida						
6.	*Limulus polyphemus*	Glycerol	−74°C	50 days	64%	Eosin dye exclusion	Behlmer and Brown, 1984

There was also no interaction between temperature and media for components other than the TFS and PBS.

Discussion

The present results indicate that there is a definite influence of freezing at different subzero temperatures on sperm viability and metabolism. In *Scylla serrata,* the viability of sperm varies between 95%–67% from −196°C to −4°C. This is in accordance with Clegg and Pickett (1966), who suggested that there is no significant decrease in fertility of bull semen stored in liquid nitrogen, in contrast to the deterioration that occurs in semen stored in dry ice. Cryopreservation of sperm or spermatophore has also been carried out in selected arthropod groups (Table III). According to Behlmer and Brown (1984), 64% of the post-thawed spermatozoa of *Limulus* at −74°C showed dye exclusion against 88% of control spermatozoa. Hughes (1973) also reported a notable decrease in viability during cryopreservation of oyster sperm.

Our experiments with freshly collected spermatophores as well as spermatophores killed intentionally, further support the utility of the dye exclusion method in determining sperm viability. Intentionally killed sperm gave a 100% dye accumulation. It is of interest to note in this context that Bishop and Walton (1968) showed an increased permeability of cell membranes at the time of cell death.

Damage caused by physical change in the membrane may be responsible for the reduced sperm viability as indicated by the work on RBC (Lovelock, 1954). During freezing and thawing, cell damage is due to the destructive action of the concentrated salt solution to which the cells are exposed when water is removed as ice. Several cryoprotectants prevent the damage to cell membrane caused during cryopreservation. Polge *et al.* (1949) first showed that the spermatozoa could be frozen and thawed without losing motility if glycerol was included in their suspending medium. In glycerol, the electrolyte concentration at temperatures below the freezing point is sufficiently reduced (Lovelock and Polge, 1954).

In this study, glycerol and DMSO + trehalose gave high sperm survival. At −4°C, DMSO and trehalose are not efficient cryoprotectants, whereas, when DMSO is combined with trehalose, sperm viability increased significantly. Successful preservation of spermatophores of shrimp in glycerol at −196°C has also been reported (Chow *et al.*, 1985). Similar results were obtained by Stephens (1986) in chicken spermatozoa where the percentage of cryoinjuries to the spermatozoa was less in glycerol and DMSO + glycerol media; when DMSO was used in the place of glycerol, for *Limulus* spermatozoa, post-thawing survival of spermatozoa was nil (Behlmer and Brown, 1984).

Cryopreserved spermatozoa of *Crassostrea virginica* in DMSO fertilized 11% of eggs exposed to them

(Hughes, 1973), whereas combining DMSO with glycine and NaHCO increased the fertility to 96% (Zell *et al.*, 1979). Asahina and Takahashi (1978) showed that DMSO exhibited protection similar to that of ethylene glycol against freezing injury, whereas at room temperature DMSO was toxic. In *S. serrata,* trehalose, when used alone, is not a good cryoprotectant; however, when combined with DMSO, the viability of sperm is significantly increased. In the ridge back shrimp *Sicyonia ingentis,* Anchordogy *et al.* (1987, 1988) also found a low sperm viability when trehalose was used as the cryoprotectant. However, when the DMSO was combined with trehalose, these authors found increased viability. Interestingly, in this shrimp, the free spermatozoa stored very well with DMSO either alone or in combination with other cryoprotectants such as trehalose, sucrose, proline, and glycerol (Anchordogy *et al.*, 1988).

Obviously, the chief concern regarding spermatozoa that have undergone cryopreservation is whether they are of a quality, in terms of viability and vitality, comparable to that of fresh spermatozoa. Although fertilization is the ultimate criterion by which the quality of post-preserved spermatozoa can be assessed, it is also presently an impractical one. It is difficult to obtain ova, as the exact period of oviposition of these crabs is unknown, which, therefore, precludes routine fertility tests. Thus the quality of cryopreserved spermatozoa was assessed in lieu of fertilization, as has been determined for *Salmo salar* spermatozoa by Yoo *et al.* (1987), by estimating the biochemical fluctuations in the preserved spermatozoa.

Biochemical alterations, if any, in the spermatophores of crustaceans that have been cryopreserved have not been previously reported. However, the protein component in the ovine seminal plasma undergoes qualitative changes during cold storage (Garner and Ehlers, 1971). Yoo *et al.* (1987) reported the loss of protein from the cryopreserved spermatozoa into the outer seminal plasma in salmon, due to leakage through the cell membrane into the outer medium. In bovine semen, Pickett and Komarek (1964) also showed leakage of lipids into the seminal plasma from the cryopreserved spermatozoa. It is not known whether any such change occurs in the protein or lipid contents relative to the sperm cells and seminal plasma of *S. serrata* during cryopreservation. In the present study, there were practical difficulties in completely isolating the spermatophores from the seminal plasma after cryopreservation; even after repeated centrifugation of seminal plasma diluted with the diluents, the granular substances of the seminal plasma adhered to the spermatophores, thus making it difficult to isolate the spermatophores. It should also be noted that arthropod spermatozoa are sensitive to centrifugation (Behlmer and Brown, 1984) and hence repeated centrifugation may cause increased leakage of organic substrates and enzymes from the sperm cells to the medium, as has also been reported by Barnes and Blackstock (1974) for cirripede semen.

Biochemical results of the present study reveal an interesting pattern of substrate use during cryopreservation. At −4°C, the total carbohydrates showed a significant decline, although protein also showed a decline. In *S. serrata,* carbohydrates formed the main substrate used during sperm maintenance within the spermatheca of the mated female (Jeyalectumie, 1989). The reduction in the carbohydrate level, when preserved at −4°C, further indicates that the metabolic activity of sperm may continue using carbohydrate substrates. The activity of the LDH also declined at −4°C. This may be due to the death of sperm cells, which accounts for 18%. According to Jacobs *et al.* (1986), the dissociation and loss of LDH activity occur at low temperatures; by 45 days of storage, human serum retained 74%, 53%, and 87% of initial activity when stored at 25°C, 4°C, and −20°C, respectively. Our preliminary observations on glycosidases activity in the seminal plasma and spermatophores of this crab using different substrates such as β-D-glucopyranoside, β-D-galactopyranoside, β-D-mannopyranoside, and α-N-Acetyl galactosaminide indicate high enzyme activity (Yu-Teh Li, T. Subramoniam, and C. Jeyalectumie, unpub. obs.). All these may indicate that sperm storage in a metabolically active condition require active substrate use by spermatozoa contained in the spermatophores.

In conclusion, our results suggest that glycerol provided the best protection for the seminal plasma and maintained a high percentage of sperm viability. DMSO is toxic at room temperature; the decreased value of spermatophore viability at −4°C when DMSO was used as the cryoprotectant, may also support this view.

Acknowledgments

This work was supported by the Department of Science and Technology, Government of India, New Delhi (Grant No. 22(8p-8)/STP-II).

Literature Cited

Anchordogy, T., J. H. Crowe, W. H. Clark Jr., and F. J. Griffin. 1987. Cryopreservation of sperm from the penaeid shrimp *Sicyonia ingentis.* Abstract from 18th Annual meeting of the World Aquaculture Society, Guayaquil, Ecuador, Jan. 1987.

Anchordogy, T., J. H. Crowe, F. J. Griffin, and W. H. Clark Jr. 1988. Cryopreservation of sperm from the marine shrimp *Sicyonia ingentis. Cryobiology* **25:** 238–243.

Asahina, E., and T. Takahashi. 1978. Freezing tolerance in embryos and spermatozoa of the sea urchin. *Cryobiology* **15:** 122–127.

Barnes, H. 1962. The composition of the seminal plasma of *Balanus balanus. J. Exp. Biol.* **39:** 345–351.

Barnes, H., and J. Blackstock. 1973. Estimation of lipids in marine animals and tissue: detailed investigation of the sulphophospho-

vanillin method for 'total' lipids. *J. Exp. Mar. Biol. Ecol.* **12:** 103–118.

Barnes, H., and J. Blackstock. 1974. Biochemical composition of the seminal plasma of the cirripede *Balanus balanus* (L) with particular respect to free amino acids and proteins. *J. Exp. Mar. Biol. Ecol.* **16:** 47–85.

Behlmer, S. D., and G. Brown. 1984. Viability of cryopreserved spermatozoa of the horseshoe crab *Limulus polyphemus*. *Int. J. Invert. Reprod. Dev.* **7:** 193–199.

Bishop, M. W. H., and A. Walton. 1968. Spermatogenesis and the structure of mammalian spermatozoa. Pp. 1–101 in *Marshall's Physiology of Reproduction*, Vol. I, Part II, A. S. Parkes, ed. Longmans, London.

Caroll, W. V., R. W. Longley, and J. H. Roe. 1956. The determination of glycogen in the liver and muscle by the use of anthrone reagent. *J. Biol. Chem.* **220:** 583–593.

Chow, S., Y. Taki, and Y. Ogasawara. 1985. Cryopreservation of spermatophore of the fresh water shrimp, *Macrobrachium rosenbergii*. *Biol. Bull.* **168:** 471–475.

Clegg, E. D., and B. W. Pickett. 1966. Effect of storage at −196°C on fertility. *A. I. Digest* Vol. XIV, No. 3.

Folch, J., M. Lees, and G. H. Bloane-Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **266:** 497–509.

Garner, D. L., and M. H. Ehlers. 1971. Effect of storage at 5°C on the Disc electrophoretic patterns of ovine and bovine seminal proteins. *J. Reprod. Fert.* **27:** 43–52.

Horton, H. F., and A. G. Ott. 1976. Cryopreservation of fish spermatozoa and ova. *J. Fish. Res. Board Can.* **33:** 995–1000.

Hughes, J. B. 1973. An examination of eggs challenged with cryopreserved spermatozoa of the American oyster, *Crassostrea virginica*. *Cryobiology* **10:** 342–344.

Ishida, T., P. Talbot, and M. Kooda-Cisco. 1986. Technique for the long term storage of lobster (*Homarus*) spermatophores. *Gamete Res.* **14:** 183–195.

Jacobs, E., P. J. Hissin, W. Propper, L. Mayer, and L. Sarkozi. 1986. Stability of lactate dehydrogenase at different storage temperatures. *Clin. Biochem.* **19:** 183–188.

Jeyalectumie, C., and T. Subramoniam. 1987. Biochemical composition of seminal secretions with special reference to LDH activity in the reproductive tissues of the field crab, *Paratelphusa hydrodromous* (Herbst). *Exp. Biol.* **46:** 231–236.

Jeyalectumie, C. 1989. Biochemical investigations on the reproductive tissues and cryopreservation of seminal secretions of a brachyuran crab *Scylla serrata* (Forskal) (Decapoda: Portunidae). PhD Thesis, University of Madras, India.

Kurokura, H., H. Kumai, and M. Nakamura. 1986. Hybridization between female red sea bream (*Pagrus major*) and male crimson sea bream (*Evynnis japonica*) by means of sperm cryopreservation. Pp. 113–116 in *The First Asian Fisheries Forum*, J. L. Macleon, L. B. Dizon, and L. V. Hosillos, eds. Asian Fisheries Society, Manila, Philippines.

Leverage, W. E., D. A. Valerio, A. P. Schultz, E. Kingsbury, and C. Dorey. 1972. Comparative study on the freeze preservation of spermatozoa of primate, bovine and human. *Lab. Anim. Sci.* **22:** 882–889.

Lovelock, J. E. 1954. Physical instability and thermal shock in red cells. *Nature* **173:** 659–661.

Lovelock, J. E., and C. Polge. 1954. The immobilization of spermatozoa by freezing and thawing and the protective action of glycerol. *Biochem. J.* **58:** 618–622.

Lowry, D. H., N. J. Rosebrough, A. L. Fair, and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.

Pickett, B. W., and R. J. Komarek. 1964. Evidence for loss of lipid from bovine spermatozoa due to freezing. *J. Dairy Sci.* **47(8):** 905–908.

Polge, C., A. U. Smith, and A. S. Parkes. 1949. Revival of spermatozoa after vitrification and dehydration at low temperatures. *Nature* **164(4172):** 666.

Roe, J. H. 1955. The determination of sugar in blood and spinal fluid with anthrone reagent. *J. Biol. Chem.* **212:** 335–343.

Sawada, Y., and M. C. Chang. 1964. Tolerance of honey bee sperm to deep freezing. *J. Econ. Entomol.* **57(6):** 891–892.

Snedecor, G. W., and W. G. Cochran. 1967. *Statistical Methods.* Oxford and IBH Publishing Co. 593 pp.

Stephens, A. J. A. 1986. A comparison of diluents for cryopreservation of rooster semen. PhD Thesis, Tamil Nadu Agricultural University, Coimbatore, India.

Winer. 1971. *Statistical Principles in Experimental Design.* McGraw Hill, Inc.

Withler, F. C. 1982. Cryopreservation of spermatozoa of some freshwater fishes cultured in South and Southeast Asia. *Aquaculture* **26:** 395–398.

Yoo, B. Y., M. A. Ryan, and A. J. Wiggs. 1987. Loss of protein from spermatozoa of Atlantic salmon (*Salmo salar L.*) because of cryopreservation. *Can. J. Zool.* **65:** 9–13.

Yoshida, A., and E. Freese. 1975. Lactate dehydrogenase from *Bacillus subtilis*. Pp. 304 in *Methods in Enzymology*, Vol. XLI, W. A. Wood, ed. Academic Press, New York.

Zaneveld, L. J. D., and K. L. Polakoski. 1977. Collection and physical examination of the ejaculate. Pp. 147–172 in *Techniques of Human Andrology*, E. S. E. Hafez, ed. Elsevier/North-Holland Biomedical Press.

Zar, J. H. 1974. *Biostatistical Analysis.* Prentice-Hall, Inc. Englewood Cliffs, N.J. 620 pp.

Zell, S. R., M. H. Bamford, and H. Hidu. 1979. Cryopreservation of spermatozoa of the American oyster, *Crassostrea virginica* Gmelin. *Cryobiology* **16:** 448–460.